

mAM Facilitates Conversion by ESET of Dimethyl to Trimethyl Lysine 9 of Histone H3 to Cause Transcriptional Repression

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Summary

Methylation of histone tails plays an important role in chromatin structure and function. Previously, we reported that ESET/SETDB1 is a histone methyltransferase (HMTase). Here, we show that SETDB1 tightly associates with the human homolog of mAM, a murine ATFA-associated factor. Although recombinant ESET can methylate lysine 9 of histone H3 (H3-K9), its activity is severely compromised when compared to that of the ESET/mAM complex. mAM stimulates ESET enzymatic activity by increasing the V_{\max} and decreasing the K_m . Importantly, mAM facilitates the ESET-dependent conversion of dimethyl H3-K9 to the trimethyl state both in vitro and in vivo. Chromatin-based transcription and ChIP analyses demonstrate that mAM enhances ESET-mediated transcriptional repression in a SAM-dependent manner, and this repression correlates with H3-K9 trimethylation at the promoter. Thus, our studies establish that promoter H3-K9 trimethylation is the cause of transcriptional repression and that mAM/hAM facilitates conversion of H3-K9 dimethyl to trimethyl by ESET/SETDB1.

Introduction

Studies in the past two years have revealed that histone methylation plays an important role in modulating chromatin structure and function (Lachner and Jenuwein, 2002; Zhang and Reinberg, 2001). Histone methylation occurs on arginine and lysine residues and is catalyzed by two distinct families of proteins, the PRMT1 family and the SET domain-containing protein family (Zhang and Reinberg, 2001). Recent studies have revealed a third family of HMTases that target lysine 79, located in the globular domain of histone H3 (Feng et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002). Accumulating evidence indicates that histone methylation plays important roles in multiple biological processes including het-

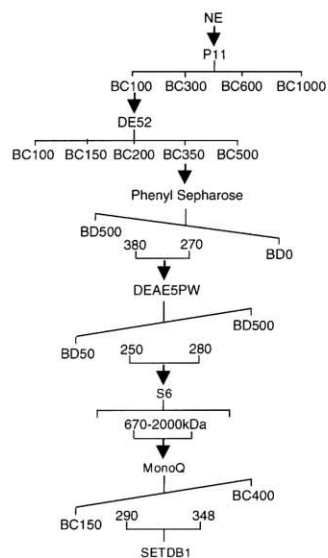
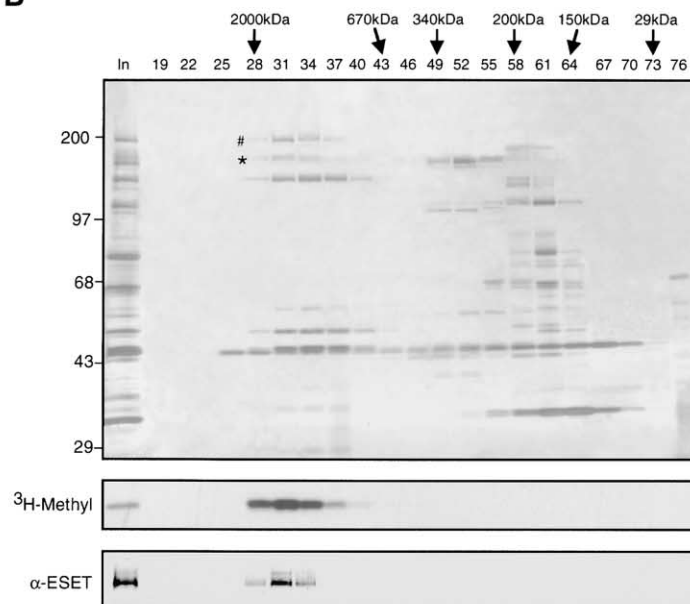
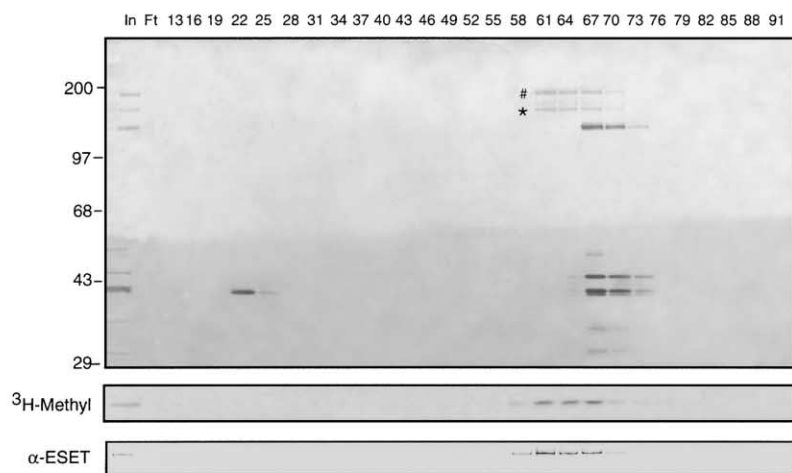
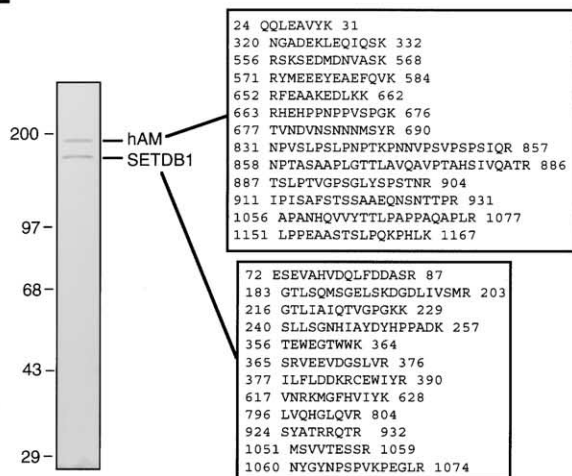
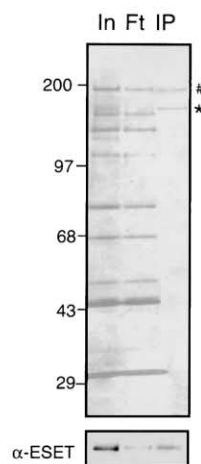
erochromatin formation, transcriptional regulation, DNA methylation, and X chromosome inactivation (Kouzarides, 2002; Lachner and Jenuwein, 2002; Zhang and Reinberg, 2001).

Thus far, six lysine residues located on histones H3 (lysines 4, 9, 27, 36, and 79) and H4 (lysine 20) have been reported to be sites of methylation. Of the different site-specific methylation events, lysine 9 methylation is the best studied due to its fundamental role in heterochromatin formation, transcriptional silencing, X chromosome inactivation, and DNA methylation (Richards and Elgin, 2002). Studies by Jenuwein and colleagues uncovered SUV39H1 and its fission yeast homolog Ctr4 as the first H3-lysine 9 (H3-K9)-specific histone methyltransferase (HMTase) (Rea et al., 2000). A link between H3-K9 methylation and transcriptional silencing was initially established by analysis of the histone methylation patterns at the fission yeast mating-type locus and the chicken β -globin locus, where H3-K9 methylation correlates with histone hypoacetylation and gene silencing (Litt et al., 2001; Noma et al., 2001). Consistent with this correlation, H3-K9 methylation was found to inhibit subsequent histone acetylation by p300 (Wang et al., 2001). However, a direct role of H3-K9 methylation in transcriptional repression has not been demonstrated.

In addition to Suv39h1 and its close relative Suv39h2 (O'Carroll et al., 2000), at least three additional mammalian proteins (G9a, ESET/SETDB1, and Eu-HMTase 1) have been reported to possess H3-K9-specific HMTase activity. Unlike the Suv39h proteins, which mainly function in heterochromatin regions (Peters et al., 2001), G9a mainly methylates histone H3 in euchromatic regions (Tachibana et al., 2001). Inactivation of G9a through targeted gene disruption in mice resulted in growth retardation and early embryonic lethality (Tachibana et al., 2002), indicating that G9a-mediated H3-K9 methylation of euchromatin is essential for early embryogenesis. Similar to G9a, ESET/SETDB1 mainly methylates histone H3 in euchromatic regions (Schultz et al., 2002). Eu-HMTase 1, which also functions in euchromatin, associates with E2F-6, Mga, and Max, and therefore is potentially involved in silencing of E2F- and Myc-responsive genes in quiescent cells (Ogawa et al., 2002).

The fact that multiple HMTases target the same lysine residue for methylation raises the question as to whether methylation on H3-K9 by different enzymes has similar consequences and how the enzymatic activities of the different HMTases are regulated to coordinate their functions. The fact that disruption of G9a and Suv39h in mice results in different phenotypes (Peters et al., 2001; Tachibana et al., 2002) indicates that these proteins perform distinct functions. Because lysine can be mono-, di-, or trimethylated and because some HMTases, such as SET7/9 and Dim5, have been shown to methylate lysines to different states (Tamaru et al., 2003; Xiao et al., 2003), it is possible that different H3-K9 methyltransferases may methylate K9 to different states. Given that transcription is affected differentially depending on the methylation state of H3-K4 (Santos-Rosa et al., 2002), it is important to determine whether

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the H3-K9 methyltransferases can methylate the same residue to different states.

Previously, we demonstrated that ESET is an H3-specific HMTase (Yang et al., 2002). To understand the function of ESET/SETDB1 and its associated HMTase activity, we searched for an *in vivo* functional partner. Here, we report the purification and characterization of a native ESET/SETDB1 complex. We demonstrate that SETDB1 tightly associates with the human homolog of mAM, a murine ATF α -associated factor with transcriptional repression activity. We found that mAM stimulates the HMTase activity of ESET through facilitating the conversion by ESET from di- to trimethyl at H3-K9. Importantly, trimethylation of H3-K9 by the ESET complex greatly impedes transcription from chromatin. Thus, our studies have uncovered mAM as the first molecule that has the ability to modulate the activity of an HMTase, and demonstrate directly that trimethylation of H3-K9 causes transcriptional repression.

Results

Purification and Identification of a SETDB1-Containing HMTase Complex

Previously, we, as well as others, demonstrated that the murine protein ESET and its human homolog SETDB1 possess histone H3-specific methyltransferase activity (Schultz et al., 2002; Yang et al., 2002). To gain insight into the function of this HMTase, we raised an antibody against the N terminus of ESET. This antibody recognized one band around 180 kDa in nuclear extracts derived from both HeLa cells and a murine cell line (Yang et al., 2002), indicating that the antibody is specific and can recognize endogenous SETDB1 in HeLa cells. To identify functional partners for SETDB1, we fractionated HeLa nuclear extracts by monitoring HMTase activity and SETDB1 protein. Using a six-column purification scheme (Figure 1A), we purified a SETDB1-containing HMTase complex. Fractionation of the partially purified sample on a Superose 6 gel filtration column followed by silver staining and enzymatic assay revealed that the enzymatic activity elutes between 670–2000 kDa and correlates with two protein bands of 200 kDa and 180 kDa, respectively (Figure 1B, top and middle panels). The 180 kDa protein band, likely to be SETDB1, was recognized by the ESET antibody (Figure 1B, bottom panel). Further fractionation of the pooled Superose 6 fractions on a Mono Q column confirmed that only the

two protein bands, one of which was recognized by the ESET antibody, copurify with the enzymatic activity (Figure 1C).

Extensive cofractionation through a variety of columns suggested that the two proteins were likely components of a stable complex. To confirm this possibility, the partially purified SETDB1 complex was immunoprecipitated by the antibody that recognizes SETDB1. The 200 kDa protein coimmunoprecipitates with the 180 kDa protein (Figure 1D, top panel). Western blot analysis confirmed that the 180 kDa protein is SETDB1 (Figure 1D, bottom panel). The association of the 200 kDa protein with SETDB1 is specific, as none of the other proteins present in the input coimmunoprecipitate with SETDB1 (Figure 1D, top panel). Given that the native complex is larger than 670 kDa (Figure 1B) while the two subunits are only about 400 kDa when added together, it is likely that the two proteins exist as a hetero-multimer.

Having established that the 200 kDa protein exists in a protein complex with SETDB1, we attempted to identify the protein by mass spectrometry. As expected, all peptides obtained from the 180 kDa protein matched SETDB1 (Figure 1E). Mass spectrometric analysis of the peptides derived from the 200 kDa protein revealed that it best matches the 609-amino acid hypothetical protein FLJ10688 that was deposited into GenBank as part of a human cDNA sequencing project. This putative partial protein sequence is highly similar to the C-terminal part of the 1306 amino acid transcriptional repressor mAM (mouse ATF α -associated Modulator) that was identified in a yeast two-hybrid screen using ATF α as a bait (De Graeve et al., 2000). Since the 609 amino acid putative protein FLJ10688 is too small to account for the observed 200 kDa mass and corresponds to the C terminus of mAM, it likely represents the C terminus of the human mAM counterpart. Analysis of multiple human EST clones and human genome sequences resulted in cloning of a 4.1 kb cDNA with an open reading frame of 1270 amino acids (accession number AY337596) that is highly related to (78% identical) the mouse protein mAM throughout the entire sequence (data not shown). Based on its similarity to mAM, we have named the human protein hAM.

mAM Stimulates the HMTase Activity of ESET

SETDB1 and its mouse homolog ESET have both been shown to contain intrinsic HMTase activity (Schultz et al., 2002; Yang et al., 2002). To assess the potential role

Figure 1. Purification of a SETDB1 Histone Methyltransferase Complex

- (A) Schematic representation of the steps used to purify the SETDB1 HMTase complex. Numbers represent salt concentrations (mM) at which SETDB1 elutes from the columns.
- (B) Silver staining of an SDS-polyacrylamide gel (top panel), HMTase activity assay (middle panel), and Western blotting analysis (bottom panel) of the fractions derived from the Superose 6 gel filtration column. The elution profile of the protein markers is indicated on top of the panel. SETDB1 is indicated by “*”, and the polypeptide that cofractionates with SETDB1 is indicated by “#.”
- (C) Silver staining of an SDS-polyacrylamide gel (top panel), HMTase assay (middle panel), and Western blotting analysis (bottom panel) of fractions derived from the Mono Q column. In and Ft represent input and flowthrough, respectively.
- (D) Affinity purification of the SETDB1 complex. An aliquot of DEAE5PW input was immunoprecipitated with anti-ESET antibody crosslinked to protein A agarose beads. Proteins that bound to the antibody (IP) were analyzed by silver staining (top panel) and Western blotting (lower panel).
- (E) Coomassie staining of a 6.5%–11% gradient SDS-polyacrylamide gel containing the purified SETDB1 HMTase complex. The identities of the polypeptides were based on mass spectrometric analysis of the indicated protein bands. The peptides obtained from each of the two proteins are listed on the right side of the gel. Numbers correspond to the amino acid number of the indicated protein.

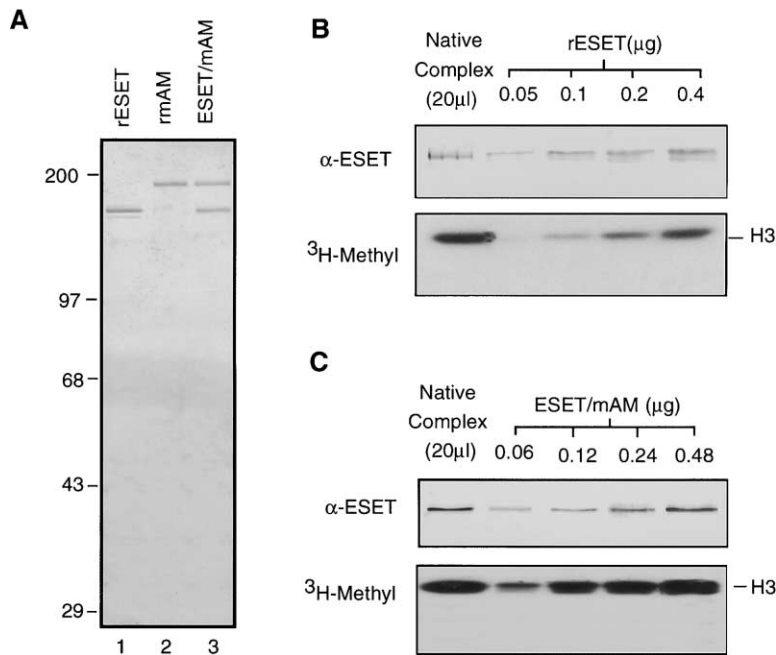


Figure 2. Reconstitution of the ESET/mAM Complex

(A) Silver staining of an SDS-polyacrylamide gel containing purified recombinant ESET, mAM, and ESET/mAM complex.

(B) Comparison of the HMTase activities of the native complex and that of recombinant ESET. The amounts of enzymes used were quantified by Western blotting (top panel). Their corresponding activities toward equal amounts of core histone substrates are shown (bottom panel).

(C) Comparison of the HMTase activities of the native complex and the reconstituted ESET complex. The amounts of enzymes used were quantified by Western blotting (top panel). Their corresponding activities toward equal amounts of core histone substrates are shown (bottom panel).

of hAM/mAM in modulating the enzymatic activity of SETDB1/ESET, we expressed and purified Flag-tagged ESET and HA-tagged mAM from baculovirus-infected SF9 cells either individually or in combination (Figure 2A). Although the recombinant ESET protein possesses histone H3 methyltransferase activity, its activity is compromised when compared with equal amounts of native complex (Figure 2B), raising the possibility that hAM/mAM may modulate the SETDB1/ESET enzymatic activity.

To explore this possibility, recombinant HA-mAM was added to the ESET HMTase assay. Surprisingly, instead of stimulating the HMTase activity, the added mAM inhibited ESET HMTase activity (data not shown). One explanation for this unexpected result is that formation of a stable, functional ESET/mAM complex requires coexpression of the two proteins. Precedent for a similar situation was reported in reconstitution of NuRD histone deacetylase activity, where stimulation of HDAC activity by MTA2 was observed only when HDAC and MTA2 were coexpressed (Zhang et al., 1999). To explore this possibility, we reconstituted the ESET/mAM complex by coinfection of SF9 cells with recombinant baculoviruses expressing Flag-tagged ESET and HA-tagged mAM and purified the ESET/mAM complex to homogeneity (Figure 2A, lane 3). Comparison of the reconstituted complex with that of the native SETDB1 complex purified from HeLa cells indicates that they possess comparable levels of HMTase activity (Figure 2C). These results collectively suggest that mAM has the ability to stimulate the HMTase activity of ESET.

Both ESET and ESET/mAM Complex Specifically Methylate H3-K9

Given that multiple lysine residues on histone H3 are susceptible to methylation (Lachner and Jenuwein, 2002; Zhang and Reinberg, 2001), the observation that

mAM stimulates the HMTase activity of ESET could be due to either or both of the following possibilities: one is that mAM modulates the substrate specificity such that the ESET/mAM complex methylates additional sites other than that methylated by ESET. The second possibility is that the ESET/mAM complex targets the same lysine residue on H3 for methylation but with an increased efficiency. To differentiate between these two possibilities, we first analyzed the effect of mAM on the substrate specificity of ESET.

Equivalent amounts of histone H3, either in complex with other core histones or in mono- or oligonucleosome form, were subjected to methylation by equal amounts of ESET alone or in a complex with mAM. While ESET and the ESET/mAM complex both can methylate H3 in core or mononucleosome forms, the enzymatic activity of ESET/mAM complex is significantly higher (Figure 3A). Interestingly, the ESET/mAM complex also possesses modest activity toward oligonucleosomal H3, which can be inhibited by the presence of histone H1 (Figure 3A, compare lanes 7 and 8).

Having established that free core histone H3 is a preferred substrate, we attempted to determine the lysine residues targeted by ESET. Thus, recombinant histone H3 was subjected to methylation by ESET in the presence of *S*-adenosyl-L-[methyl-³H]methionine (³H-SAM). Microsequencing of the methylated H3 followed by liquid scintillation counting revealed that lysine 9 is the only residue methylated by ESET (Figure 3B). A similar result was obtained when the native SETDB1 complex was used (data not shown). To confirm that lysine 9 is the only site methylated by this enzyme, histone H3 mutants were used as substrates. Results shown in Figure 3C indicate that while mutation of lysine 9 completely eliminated the ability of H3 to serve as a substrate (lane 3), mutation of any of the other potential methylation sites did not significantly affect the ability of H3 to serve as a substrate regardless of whether ESET, the reconsti-

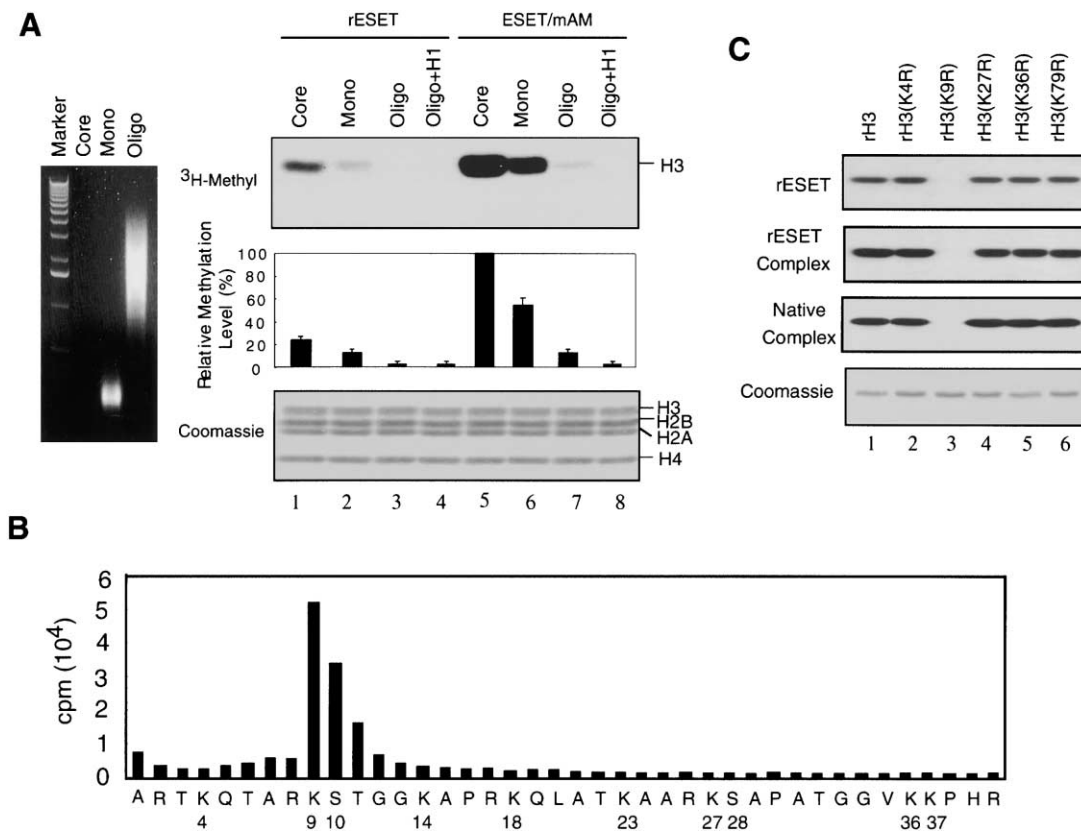


Figure 3. Substrate and Site-Specificity of Recombinant ESET and ESET Complex

(A) ESET and ESET complex prefer nonnucleosomal histone H3 substrates. Left panel shows an ethidium bromide-stained agarose gel containing core histones, mononucleosomes, and oligonucleosomes as indicated. Equal amounts of ESET, alone or in complex with mAM, were used to methylate equal amounts of histones (bottom panel) in different contexts. The top panel is an autoradiograph of the bottom panel. Quantification of two independent experiments is shown in the middle panel. Variations between the two experiments are indicated by error bars.

(B) Lysine 9 of H3 is the major methylation site of SETDB1/ESET. Recombinant H3 that was methylated by the native complex was blotted onto PVDF membrane and subjected to N-terminal automated sequencing. ³H-radioactivity eluted from each cycle was quantitated by scintillation counting. The amino acids identified at each cycle of microsequencing are listed on the bottom.

(C) HMTase assays using recombinant H3 proteins with mutations at different lysine residues as substrates. The lysine residues that were mutated are indicated on the top of the panel. Enzymes used in the assay are indicated on the left of the panel. Coomassie staining shows that an equal amount of substrate was used in the assay.

tuted ESET/mAM complex, or the native SETDB1 complex was used as the enzyme source. Thus, we conclude that mAM does not alter the substrate specificity of ESET.

mAM Stimulates the HMTase Activity of ESET by Increasing V_{max} and Decreasing K_m

To address the possibility that mAM stimulates the HMTase activity of ESET by altering the reaction kinetics, we determined the reaction kinetics using recombinant ESET and the ESET/mAM complex. A time course of HMTase activity assays established that the reaction is linear over 30 min (data not shown). Thus, all reactions were performed for 15 min. By varying the SAM concentration, we determined the reaction velocities for both recombinant ESET (Figure 4A) and ESET/mAM complex (Figure 4B). The maximal velocity (V_{max}) and the Michaelis-Menten constant (K_m) were then determined by a double-reciprocal plot. This analysis revealed that the K_m and V_{max} for ESET are 1.78 μ M and 167 methyl groups/

min/ESET, respectively, while the K_m and V_{max} for ESET/mAM complex are 1.27 μ M and 455 methyl groups/min/complex, respectively. Based on these data, we conclude that mAM stimulates the ESET HMTase activity by increasing the V_{max} 2.7-fold and decreasing the K_m 1.4-fold. Since V_{max} measures the turnover number and K_m represents the dissociation constant of the enzyme-substrate complex, our results indicate that mAM stimulates the HMTase activity of ESET mainly through an increase in the turnover rate of the reaction.

mAM Facilitates Conversion by ESET of H3-K9 from Di- to Trimethyl

Given that lysine methylation can exist in three different states (mono-, di-, and trimethylated), we attempted to address whether the stimulation occurs at a particular step. To this end, we compared the ability of ESET and the ESET/mAM complex to methylate H3 peptides that are either unmethylated or dimethylated at lysine 9. At the enzyme concentrations tested and using an unmeth-

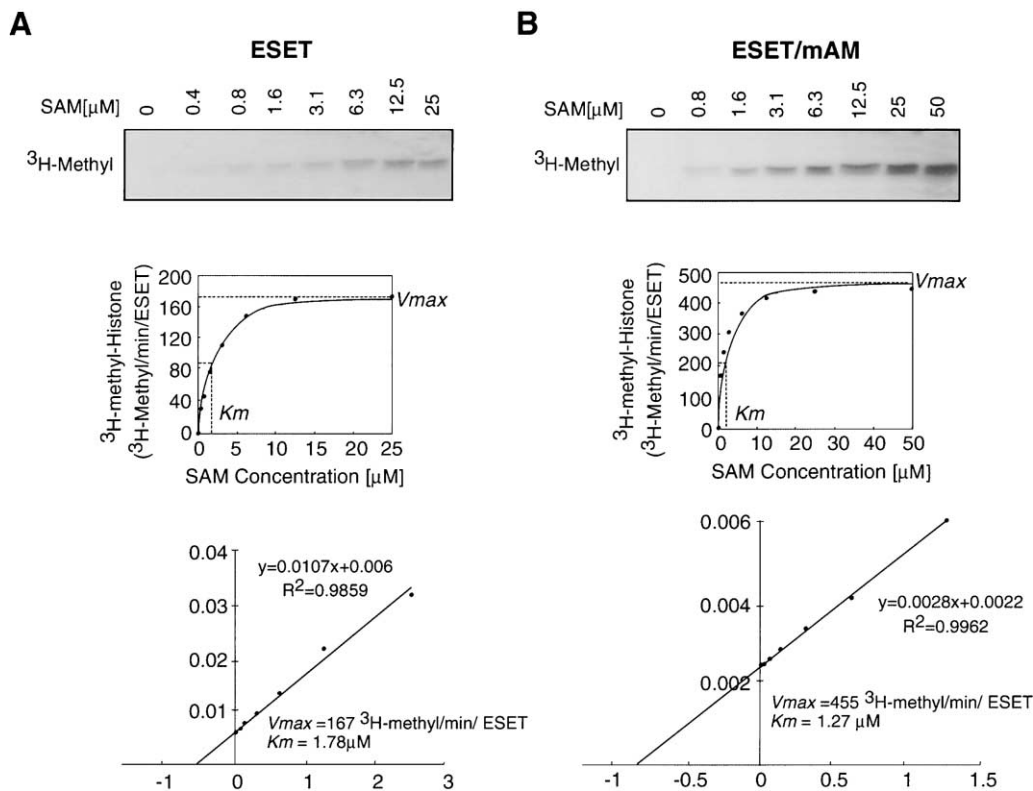


Figure 4. Kinetic Analysis of the HMTase Reactions Catalyzed by ESET and ESET Complex Using Core Histones as the Substrate. Representative autoradiographs are shown in the top panels, and quantification of two independent experiments is shown in the middle panels. Double-reciprocal plots of the reactions catalyzed by ESET and ESET complex are shown in the bottom panels. K_m and V_{max} values were derived from the double-reciprocal plots. (A) and (B) use ESET and ESET/mAM complex as enzyme, respectively.

ylated peptide substrate, the ESET/mAM complex is about 2-fold more active than ESET alone (Figure 5A, compare filled circles with filled squares). However, efficient methylation of an H3-K9-dimethylated peptide by ESET requires association with mAM (compare open circles with open squares). To ascertain that mAM mainly affects the efficiency of ESET in converting dimethyl to trimethyl, the data in Figure 5A are presented in Figure 5B using an alternative method. The total incorporated radioactivity for mono- and dimethylation of the unmethylated peptide was derived by deducting the radioactivity observed with the dimethylated peptide as a substrate. From this analysis, it is clear that while ESET and the ESET/mAM complex have similar efficiencies in methylating H3-K9 to mono- and dimethyl states (Figure 5B, compare open and filled circles), they differ significantly in their abilities to convert the dimethyl H3-K9 to trimethyl (compare open and filled squares). The ability of mAM to facilitate conversion by ESET of H3-K9 from dimethyl to trimethyl was also confirmed by a time course study (Figure 5C, compare open with filled squares).

To further verify the above result, we extended the peptide studies to recombinant H3. After methylation of recombinant H3 with amounts of ESET or ESET/mAM complex that transferred equal numbers of methyl groups (Figure 5D, lanes 2 and 3 in second panel), the

samples were divided into three parts for fluorogram and Western blotting with antibodies that recognize di- or trimethyl H3-K9. Results shown in Figure 5D (bottom two panels) indicate that ESET alone mainly methylates H3-K9 to the dimethyl state, while the ESET/mAM complex mainly methylates H3-K9 to the trimethyl state. Collectively, the above results allow us to conclude that mAM facilitates the ability of ESET to convert H3-K9 from the dimethyl to the trimethyl state in vitro.

To assess the in vivo relevance of the above finding, we asked whether the methylation state of the bulk H3-K9 changes when SETDB1 and hAM are knocked down using the siRNA approach. Toward this end, an RNA oligonucleotide corresponding to amino acids 803–808 of SETDB1 was synthesized and transfected into both 293T and HeLa cells. As controls, parallel mock transfections were also performed. To increase the knock-down efficiency, a second transfection was performed 24 hr after the initial transfection. Interestingly, transfection of siRNA for SETDB1 resulted in cell death in both cell lines (Figure 6A). To assess the effect of the transfection on SETDB1 and H3-K9 methylation levels, the cells still alive after 3 days of transfection were collected and analyzed by Western blotting. Results shown in Figure 6B indicate that transfection of siRNA resulted in significant reduction of SETDB1 in both cell lines while having no effect on an unrelated protein SUZ12 (top two panels). Consistent with the fact that SETDB1 is an H3-

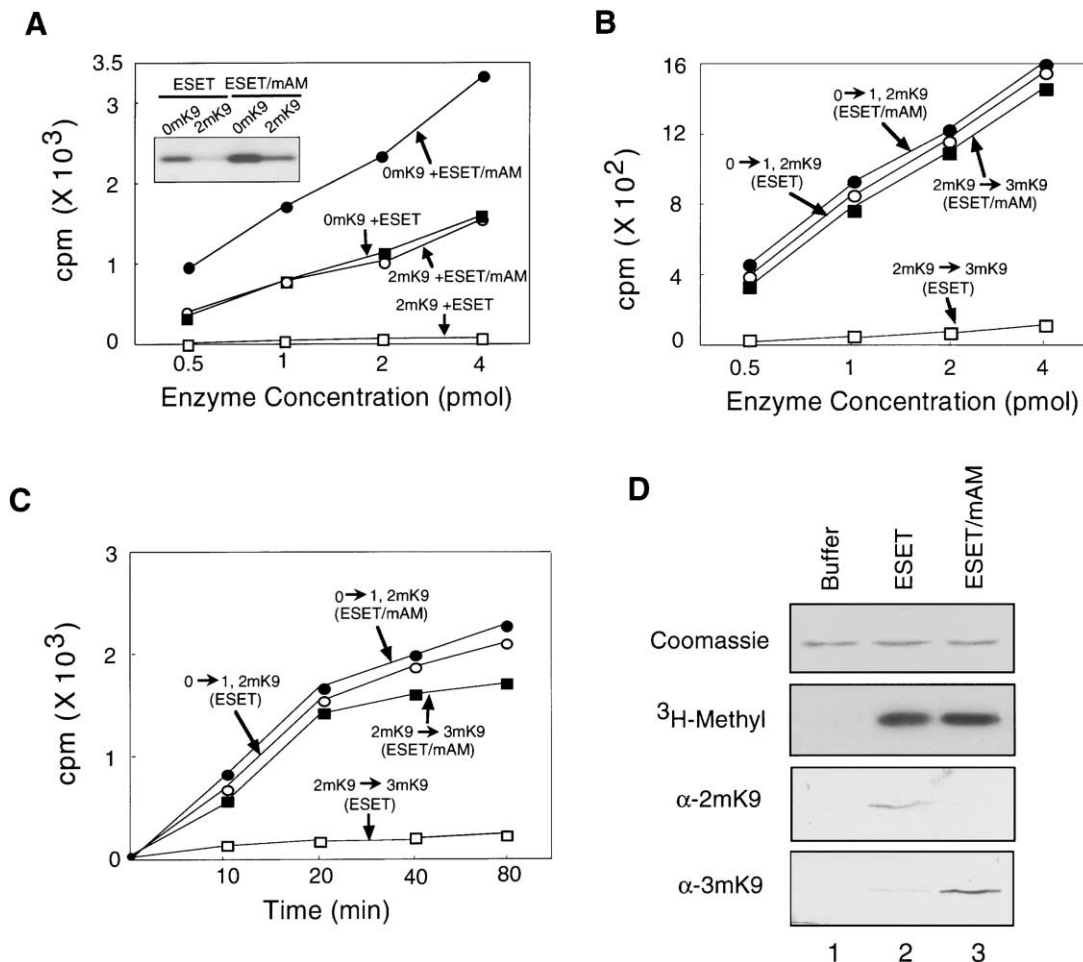


Figure 5. mAM Facilitates Conversion of Di- to Trimethyl Lysine at H3-K9 by ESET

(A) Comparison of the efficiencies of ESET and the ESET/mAM complex in methylating unmodified or dimethylated H3 peptides (aa 1–21). Different concentrations of enzymes were incubated with peptide substrates (9 μ M) in the presence of 3 H-SAM at 30°C for 15 min. After resolving the proteins on an SDS-polyacrylamide gel, the dried gel slices were quantified and plotted. A representative autoradiograph of an SDS-polyacrylamide gel is presented in the insert.

(B) Comparison of the ability of ESET and ESET/mAM complex to methylate H3-K9 to different methylated states. Data presented were derived from (A) (see text).

(C) A time course comparison of the efficiency of ESET and ESET/mAM complex to methylate H3-K9 to different methylated states. Reactions were performed with an enzyme concentration of 2 pmol. Data were derived by methods similar to those used in (B).

(D) Equal amounts of HMTase activity of ESET alone or in a complex with mAM were used to methylate equal amounts of recombinant histone H3 (top panel). The reactions were divided into three parts for autoradiography (second panel) and Western blotting (bottom two panels) using antibodies specific for dimethylated and trimethylated lysine 9, respectively.

K9-specific HMTase, siRNA-mediated knock-down of SETDB1 resulted in decreased levels of di- and trimethyl-K9 (Figure 6B and data not shown).

To further verify the above results, we attempted to suppress SETDB1 expression using a modified DNA vector-based RNAi approach (Brummelkamp et al., 2002; Sui et al., 2002). DNA constructs expressing siRNAs that target two different regions of SETDB1 (aa 803–808 and 384–389) were transfected into HeLa and 293T cells in parallel with an empty vector. Transfected cells were selected by culturing the cells in the presence of puromycin. Six days after selection, the SETDB1 level and H3-K9 methylation state were analyzed by Western blotting. Consistent with the siRNA oligonucleotide-mediated SETDB1 knock-down results (Figure 6B),

transfection of two different constructs expressing siRNAs for SETDB1 resulted in a significant reduction in the SETDB1 level and concomitant decreases in the levels of di- and trimethyl-K9 (Figure 6C). Consistent with the observation that the SETDB1 knock-down resulted in cell death, further selection of the cells in the presence of puromycin did not result in stable SETDB1 knock-down cell lines. Indeed, neither the SETDB1 level nor the H3-K9 methylation levels changed in cells transfected with vectors expressing siRNAs for SETDB1 relative to that of an empty vector after 3 weeks of puromycin selection (data not shown). Our explanation is that cells expressing siRNA for SETDB1 die while the surviving cells only express the puromycin resistance gene.

To address whether hAM is capable of facilitating

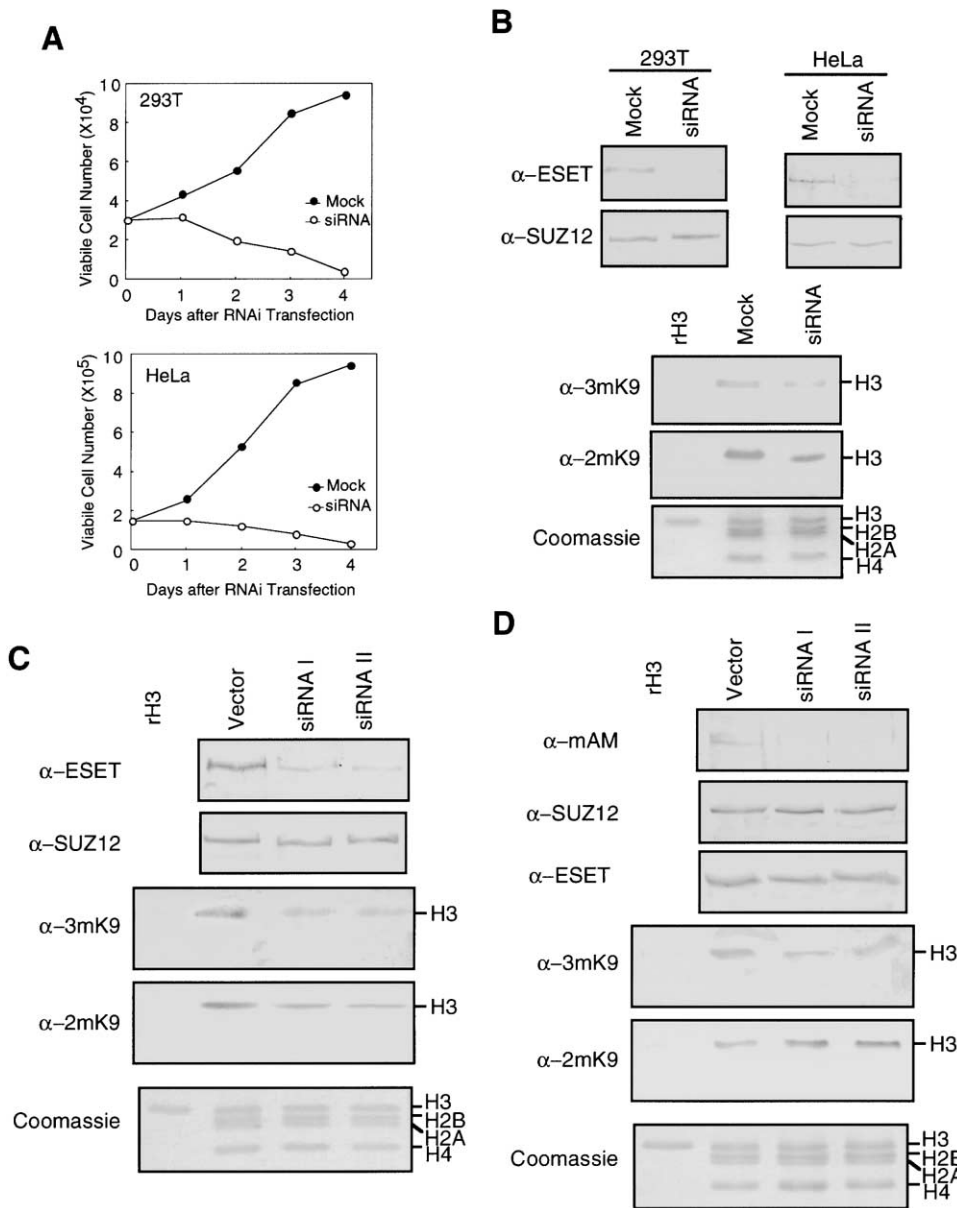


Figure 6. Knock-Down of SETDB1 and hAM by siRNA Affects H3-K9 Dimethyl and Trimethyl Levels

(A) Knock-down of SETDB1 results in cell death. Survival curves of 293T and HeLa cells after mock and SETDB1 siRNA transfections. Viable cells were counted by trypan blue staining at different days after the second transfection (see Experimental Procedures).

(B) Western blot analysis of total cell lysates derived from mock and siRNA transfected cells (top panels). Equal amounts of histones extracted from mock and siRNA transfected 293T cells were blotted with antibodies specific for di- or trimethylated H3-K9 (bottom panels). Antibodies used are indicated on the left side of the panels.

(C) Western blot analysis of total cell lysates or histones derived from 293T cells transfected with empty vector or vectors expressing siRNAs targeted to two regions of SETDB1 after 6 days of selection (top four panels). Equal loading of histone H3 was verified by Coomassie staining of a parallel gel. The labeling is the same as that in (B).

(D) The same as that in (C) except that hAM was the target for knock-down by DNA vector-based siRNA.

H3-K9 trimethylation by SETDB1 in vivo, a similar DNA vector-based RNAi approach was used to knock-down hAM. Results shown in Figure 6D indicate that knock-downs of hAM with two siRNA-expressing vectors that target different regions of hAM (aa 506–511 and 1037–1042) each resulted in a significant reduction of the hAM level concomitant with an increased level of dimethyl-K9 and a decreased level of trimethyl-K9. However, the SETDB1 level was not affected by hAM knock-down

(Figure 6D). These results, in combination with the data shown in Figure 5, allow us to conclude that mAM/hAM facilitates ESET/SETDB1-mediated methylation of H3-K9 to the trimethyl state both in vitro and in vivo.

mAM Enhances Transcriptional Repression by ESET Concomitant with Promoter H3-K9 Trimethylation
Having established that mAM/hAM facilitates conversion by ESET/SETDB1 from dimethyl-H3-K9 to trimethyl-

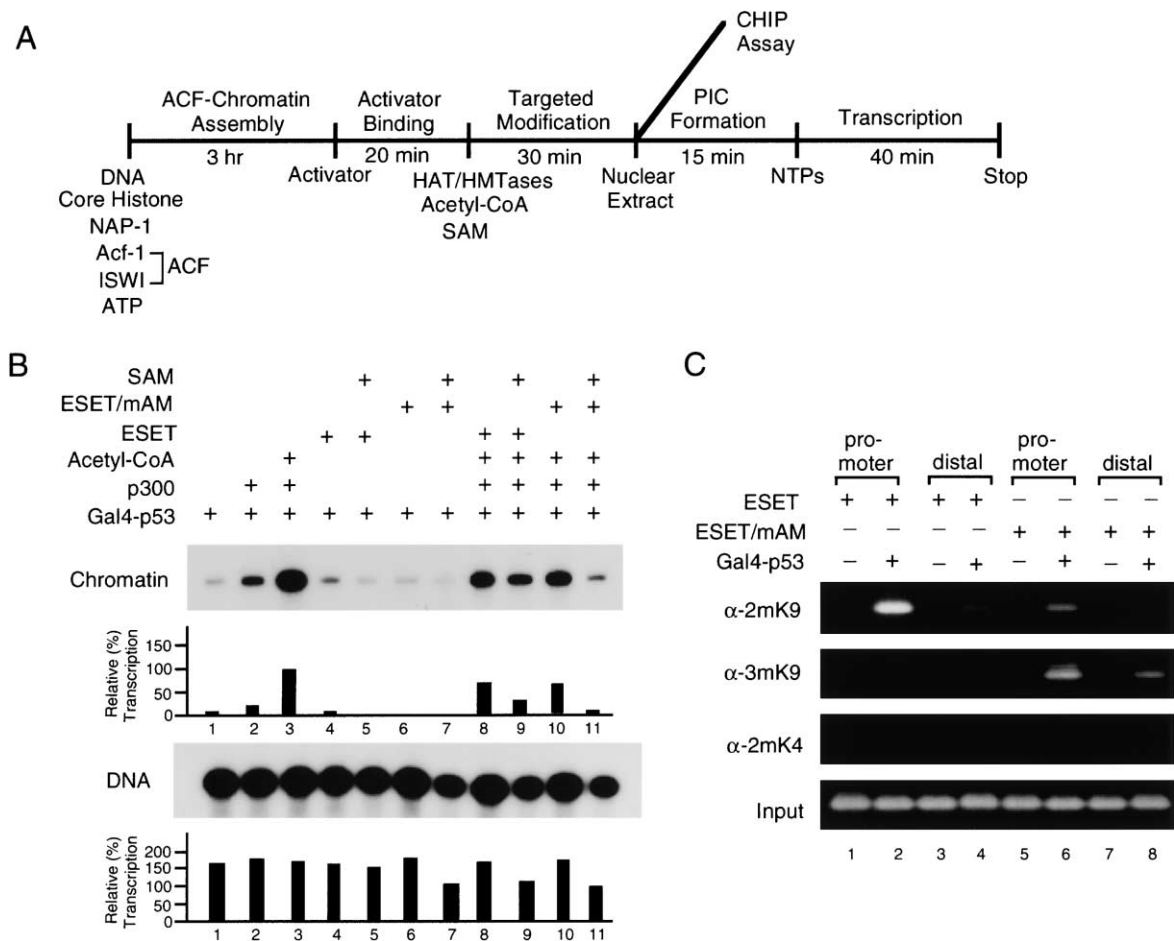


Figure 7. mAM Enhances ESET Transcriptional Repression by Facilitating Promoter-Specific Trimethylation on H3-K9

(A) Scheme of transcription and ChIP assays.

(B) Representative transcription and quantification results using chromatin and naked DNA templates. “+” indicates the presence of a particular factor in the reaction. For comparison of repression activity, equivalent amounts of ESET and ESET/mAM complex, based on the transfer of equal numbers of methyl groups to H3-K9, were used in transcription reactions. Experiments were repeated three times with comparable results. Similar results were also obtained when Gal4-p53 was replaced by Gal4-VP16.

(C) Representative results of ChIP analysis of the histone methylation status in promoter and distal regions. “+” and “-” indicate the presence or absence of a particular factor. Antibodies used in the ChIP assay are indicated on the left of the panel. Experiments were repeated twice with comparable results.

H3-K9, we attempted to address the functional consequence of this conversion. Toward this end, recombinant ESET or ESET/mAM complex was used in a reconstituted chromatin transcription system in which transcription activation (here by Gal4-p53) is dependent upon p300 and acetyl-CoA (An et al., 2002). As diagrammed in Figure 7A, chromatin assembly, remodeling, and activator binding were achieved before addition of the histone-modifying enzymes p300 and either ESET or the ESET/mAM complex. After histone modification, nuclear extract was added to allow preinitiation complex formation, and transcription was initiated by NTP addition. Similar to previous results (An et al., 2002), Gal4-p53-activated transcription was stimulated by p300 and acetyl-CoA (Figure 7B, top two panels, lanes 1–3). Since the low level of transcription in the absence of coactivator p300 precluded a clear demonstration of the repression activity of ESET or the ESET/mAM complex (lanes 4–7), the effect of ESET and the ESET/mAM complex on transcription was analyzed in the presence of p300

(lanes 8–12). In the absence of the methyl donor SAM, only a modest repression activity was observed for both ESET and the ESET/mAM complex (Figure 7B, top two panels, compare lanes 8 and 10 with 3). In the presence of SAM, however, a much more pronounced level of repression (up to 20-fold for the ESET/mAM complex) was achieved (compare lanes 9 and 11 with 3). No significant differences in transcription were detected in parallel experiments using a naked DNA template (Figure 7B, bottom two panels). Similar results were also obtained when Gal4-p53 was replaced by Gal4-VP16 (data not shown). The SAM-dependent effect strongly suggests that the repression activity is dependent on the HMTase activity of ESET or the ESET/mAM complex. Moreover, the observation that mAM enhances ESET repression activity, which requires the presence of SAM, strongly argues that H3-K9 trimethylation is the cause of the repression.

The SAM-dependent transcriptional repression by ESET or the ESET/mAM complex in the above chroma-

tin-based transcriptional assay appears contrary to the demonstration that neither ESET nor ESET/mAM has significant HMTase activity toward oligonucleosomal histone substrate (Figure 3A). However, it is possible that chromatin remodeling by ACF (present in the transcription assay) coupled with binding of activators may open up the promoter region and allow nucleosomal histone methylation by ESET and ESET/mAM complex. To test this possibility, the methylation state of H3-K9 in the promoter region and in a distal region 2 kb downstream were analyzed by chromatin immunoprecipitation (ChIP) assays. These results clearly demonstrate oligonucleosomal H3-K9 methylation both by ESET and by the ESET/mAM complex (Figure 7C). In addition, ESET activity is restricted to dimethylation whereas the ESET/mAM complex activity results primarily in trimethylation (compare lanes 2 and 6). Importantly, methylation is largely restricted to the promoter region and is completely dependent upon the presence of the activator. Parallel ChIP assays using a dimethyl-H3-K4 antibody demonstrate that H3-K4 is not methylated, verifying the specificity of both the enzyme and the ChIP assay. Collectively, the above results confirm that ESET and ESET/mAM complex are capable of methylating nucleosomal histone at H3-K9 and that the consequence of this modification is transcriptional repression. Moreover, the mAM-enhanced transcriptional repression by ESET is dependent on its ability to facilitate conversion from di- to trimethyl at H3-K9.

Discussion

ESET/SETDB1 Functions as a Heteromultimer and Can Be Targeted to Euchromatic Regions by Multiple Transcription Factors

ESET was initially identified in a yeast two-hybrid screen aimed at identifying functional partners for the ETS family of transcription factor ERG (Yang et al., 2002). The presence of evolutionarily conserved SET, preSET, and postSET domains implicated in histone methylation (Rea et al., 2000) in ESET prompted us to test for an intrinsic HMTase activity. As a result, we demonstrated that ESET specifically methylates histone H3 (Yang et al., 2002). Independent studies using the KRAB-ZFP corepressor KAP-1 as a bait identified the human homolog of ESET, named SETDB1, as an H3-K9 methyltransferase (Schultz et al., 2002). Importantly, SETDB1 colocalizes with the heterochromatin protein HP1 in nonpericentromeric euchromatic regions and both proteins are recruited to an integrated reporter containing KRAB binding sites, thus suggesting that SETDB1 plays an important role in euchromatic gene silencing (Schultz et al., 2002). In this study, we demonstrate that ESET/SETDB1 associates with mAM/hAM *in vivo* and that the two proteins likely function as a heteromultimer as judged by the sizes of the native human complex (Figure 1B) and the reconstituted mouse complex (data not shown). Comparison of the enzymatic activities of the recombinant ESET and the ESET/mAM complex revealed that association of mAM with ESET has functional consequences. mAM stimulates ESET enzymatic activity, and this effect requires incorporation of the two proteins into one protein complex (Figure 2). Significantly, mAM facilitates ESET to convert di- to trimethyl at H3-K9 both *in vitro* and *in vivo* (Figures 5 and 6). This capability is linked to its capacity to enhance transcriptional repression by ESET on a chromatin template (Figure 7).

in vivo (Figures 5 and 6). This capability is linked to its capacity to enhance transcriptional repression by ESET on a chromatin template (Figure 7).

In addition to modulating the enzymatic activity of ESET and enhancing its transcriptional repression activity, mAM is also likely to bridge interactions of ESET with certain transcription factors. mAM was initially identified in a yeast two-hybrid screen using ATF α , a member of the CREB/ATF family of transcription factors, as a bait (De Graeve et al., 2000). mAM has been reported to possess a weak ATPase activity. Although this ATPase activity may potentially contribute to the ability of the ESET/mAM complex to methylate nucleosomal histones, its exact function is currently unknown. Protein-protein interaction studies indicate that mAM is able to interact with components of the basal transcription machinery including TFIIE, TFIIH, and RNAPII (De Graeve et al., 2000), raising the possibility that this might contribute to its repression activity. However, the SAM-dependent enhancement of transcriptional repression and promoter-specific trimethylation on H3-K9 strongly argue that histone methylation is a major cause of the observed transcriptional repression (Figure 7). Whether ESET/mAM can also methylate components of the basal transcription machinery remains to be determined. Thus far, at least three transcription factors or corepressors that belong to distinct protein families have been demonstrated to be capable of interacting with ESET/SETDB1 or mAM. These proteins include the ETS family member ERG (Yang et al., 2002), the KRAB-ZFP corepressor KAP-1 (Schultz et al., 2002), and the CREB/ATF family member ATF α (De Graeve et al., 2000). These observations suggest that the ESET/mAM complex is likely to be involved in the regulation of a variety of genes targeted by the different transcription factors. This is consistent with the fact that SETDB1/ESET is widely distributed throughout the nucleus (Schultz et al., 2002). Given the existence of at least four HMTases that methylate H3-K9, it will be important to determine how the different HMTases with the same site specificity are targeted to different genes to perform their functions.

Transcriptional Repression by H3-K9 Methylation Is Dependent on the Methylation State

Histone methylation plays an important role in transcriptional regulation (Kouzarides, 2002; Zhang and Reinberg, 2001). Unlike histone acetylation, which generally correlates with gene activation (Roth et al., 2001), the consequence of histone methylation appears to be site dependent. For example, H3-K4 methylation correlates with transcriptional activation while H3-K9 methylation correlates with transcriptional repression (Litt et al., 2001; Noma et al., 2001). However, recent studies indicate that the effects of histone lysine methylation on transcription are likely to be even more complicated. Studies in budding yeast, using antibodies that discriminate between the di- and trimethylated states, indicate that while dimethylation of H3-K4 can occur at both inactive and active euchromatic genes, trimethyl-H3-K4 is present exclusively at active genes (Santos-Rosa et al., 2002). This observation indicates that the consequences of histone methylation for gene expression are not only site dependent, but also are methylation state

dependent. Consistent with this notion, we and others have recently demonstrated that H3-K27 trimethylation but not dimethylation is involved in X inactivation (Plath et al., 2003; Silva et al., 2003). Using a chromatin-based transcriptional system coupled with ChIP assays, we have now demonstrated that methylation on H3-K9 is the cause of transcriptional repression. Consistent with this notion, targeting of an HMTase-deficient SUV39H1 mutant is less efficient in transcriptional repression than that of its wild-type counterpart (Snowden et al., 2002). Significantly, we demonstrate that promoter H3-K9 trimethylation results in much more potent transcriptional repression than promoter H3-K9 dimethylation. Thus, methylation state-dependent "read out" appears to be a general phenomenon.

Why do cells need different HMTases to methylate H3-K9? One explanation is that different enzymes are targeted to different chromosomal domains and/or genes. Alternatively, different enzymes may have different preferences toward non-, mono-, and dimethylated substrates. Previous studies using recombinant SUV39H1 indicate that dimethylated H3 peptide is a poor substrate, suggesting that SUV39H1 alone may not be able to trimethylate histone H3 (Rea et al., 2000). Whether SUV39H1 is capable of trimethylating H3 when it associates with its *in vivo* functional partner remains to be determined. Our preliminary analysis of G9a null ES cells indicates that while the loss of G9a greatly affects the bulk H3-K9 dimethylation level, the trimethylation level does not appear to be affected (data not shown), indicating that G9a may not be able to trimethylate H3-K9. Given the sequence and structural similarities between Eu-HMTase 1 and G9a, Eu-HMTase 1 is unlikely to catalyze H3-K9 trimethylation. Since most, if not all, ESET is tightly associated with mAM *in vivo*, it is likely that a major function of ESET is to trimethylate H3-K9. Whether it is the only enzyme that catalyzes H3-K9 trimethylation remains to be determined. The identification of mAM/hAM as an ESET/SETDB1 functional partner and the demonstration that mAM enhances the ability of ESET to repress transcription through trimethylation on H3-K9 provide direct evidence that H3-K9 trimethylation is the cause of transcriptional repression.

It worth noting that while some mammalian HMTases such as SET7/Set9 (Nishioka et al., 2002a; Wang et al., 2001) and SET8/PR-Set7 (Fang et al., 2002; Nishioka et al., 2002b) were purified as single subunit enzymes, a few others such as EZH2 (Cao et al., 2002; Kuzmichev et al., 2002), its *Drosophila* counterpart E(Z) (Czernin et al., 2002; Muller et al., 2002), and ESET have been found to function as protein complexes *in vivo*. The other subunits in these enzyme complexes likely modulate the enzymatic activity or substrate specificity. They also likely play important roles in targeting the HMTases to specific genes by mediating interactions between the enzymes and specific transcription factors. The challenge for us in the future is to identify specific genes targeted by the various enzymes or enzyme complexes.

Experimental Procedures

Purification of the Native SETDB1 Complex, Recombinant ESET, mAM, and ESET/mAM Complex

Purification of the native SETDB1 complex is outlined in Figure 1A. A detailed description of the purification process is available in the

Supplemental Data (<http://www.molecule.org/cgi/content/full/12/2/475/DC1>). Recombinant ESET, mAM, and ESET/mAM complex were expressed in SF9 cells. Purification was achieved using a combination of ion exchange, gel filtration, and affinity columns. A detailed description of the purification process is available in the Supplemental Data.

HMTase Assay, Western Blotting, Protein Identification, and Methylation Site Determination

Histone methylation and Western blot analyses were performed as previously described (Wang et al., 2001). Substrates used for the HMTase assays included histone octamer, mononucleosomes, and oligonucleosomes that were purified as described (Fang et al., 2002). Protein identification and methylation site determination were also performed as previously described (Wang et al., 2001). Wild-type and mutant recombinant histone H3 were purified as previously described (Cao et al., 2002). Peptides and the H3-K9 dimethyl-specific antibodies were purchased from Upstate Biotechnology (USA). ESET and trimethyl-specific H3-K9 antibodies were previously described (Yang et al., 2002). Antibodies against mAM were produced in rabbits using the first 320 amino acids as antigen.

Kinetic Analysis of the HMTase Reactions

Methylation reactions were performed with different concentrations of ³H-SAM. The reactions were allowed to proceed for 15 min and were terminated by the addition of SDS sample buffer. Histones were separated by 18% SDS-PAGE, and gel pieces containing histone H3 were excised and quantified by scintillation counting. A histone sample with a known amount of radioactivity (cpm) was run at the same time to calibrate the methyl group incorporation into histones.

Cell Viability and Knock-Down Assays

HeLa and 293T cells were cultured in DMEM media supplied with 10% FBS. siRNA, with the sequence of 5'-AAGUUCGGCUCAGCU AUUCA-3' (Dharmacon), was transfected into cells using Oligofectamine (Invitrogen) following the manufacturer's instruction. The transfection was repeated 24 hr after the initial transfection. Cell viability was determined by trypan blue staining. Three days after transfection, cells were harvested and total cell extracts and histones were prepared for Western blotting as previously described (Wang et al., 2001). For DNA vector-based knock-down, the desired 19 bp stem-loop RNAs were expressed from the pHTPsiRNA vector (a gift from Bill Reed at UNC-Chapel Hill) driven by the human H1 gene promoter. The vector also contains an SV40 promoter that drives transcription of a puromycin N-acetyl-transferase gene conferring puromycin resistance. Vectors expressing the following hairpin RNAs were transfected into HeLa and 293T cells by Effectene (Invitrogen). Transfected cells were selected with 2 μ g/ml (HeLa) or 4 μ g/ml (293T) puromycin for 6–7 days before analysis. The two siRNAs that target SETDB1 are: 5'-GTTCCGGCTACAGCTATTCATT CAAGAGATGAATAGCTGTAGCCGAAC-3' and 5'-ATGTGAGTGGAT CTATCGTTCAAGAGACGATAGATCCACTCACATC-3'. The two siRNA that target hAM are: 5'-CATCTCTCCTTCCAATCGATTCAAGAGAT CGATTGGAAGGAGAGATG-3' and 5'-ATCACACTCCTGTATCAACT TCAAGAGAGTTGATACAGGAGTGTGAT-3' (targeted sequences are underlined).

Transcription and ChIP Assays

The steps involved in transcription and ChIP assays are outlined in Figure 7A. For *in vitro* transcription, chromatin template (40 ng) was preincubated with Gal4-p53 (20 ng) and histone-modifying factors (10 ng p300, 30 ng ESET, 20 ng ESET/mAM complex) prior to the sequential addition of nuclear extract (for preinitiation complex formation) and the four ribonucleoside 5'-triphosphates (for initiation of transcription) as described (An et al., 2002). *In vitro* ChIP assays were performed essentially as described (An et al., 2002), except that DNA sequences in the immunoprecipitates were monitored by PCR amplification using primers that flank the promoter (5' primer, 5'-CTCGGATCCAGCGAGCTCGAGTGC-3'; 3' primer, 5'-AGTGAG GACGAACGCGCCCCACC-3') or primers for a distal region located 2 kb downstream from the promoter (5' primer, 5'-GACGCTCAGTG GAACGAAAATCA-3'; 3' primer, 5'-GGAGGCGGATAAAGTTGCA

GGACC-3'). PCR analysis with identical primers on input chromatin confirmed that equal chromatin amounts were used for all reactions.

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